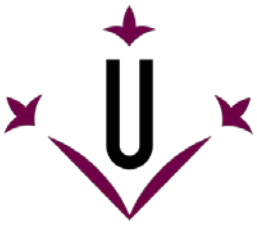

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Toxicity and residual activity of spinetoram to neonate larvae of *Grapholita molesta* (Busck) and *Cydia pomonella* (L.) (Lepidoptera: Tortricidae): semi-field and laboratory trials

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Abstract

Spinetoram is a fermentation insecticide, derived from the actinomycete *Saccharopolyspora spinosa*. It works by disrupting the GABA-gated chloride channels and by causing persistent activation of insect nicotinic acetylcholine receptors. This study aimed to evaluate the efficacy of spinetoram for control of neonate larvae of both oriental fruit moth (OFM) *Grapholita molesta* (Busck) and codling moth (CM) *Cydia pomonella* (L.) in semi-field and laboratory trials. OFM and CM neonate larvae responded similarly to spinetoram, which showed high efficacy on both species. In semi-field experiments, regression analysis of the percentage of damaged fruits as a function of

days after treatment showed a better performance of the highest spinetoram dose (10 g a.i./hl) in comparison with the maximum recommended field dose of the reference product emamectin benzoate (2.85 g a.i./hl). Surface-treated diet assays revealed LC_{50} values of 6.59 and 8.44 ng a.i./cm^2 for neonate larvae of OFM and CM larvae, respectively. High percentages of mortality were recorded on both species after 24-hour exposure to treated diet. For these reasons spinetoram could be considered a valuable tool in IPM strategies for OFM and CM control.

Keywords: Oriental fruit moth, Codling moth, Residual activity, Baseline susceptibility, IPM, Orchards, Arthropod bioassays.

1. Introduction

The codling moth - *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) - is a key pest of pome fruit and walnut in all temperate regions of the world, except Japan, Korea and Brazil. The oriental fruit moth - *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) - is a worldwide key pest of stone fruits, but occurs widely also on pome fruits (Kirk et al., 2013; Lu et al., 2014; Myers et al., 2007; Najar-Rodriguez et al., 2013; Natale et al., 2003; Rothschild and Vickers, 1991; Wearing et al., 2001).

Although mating disruption is a useful management tool (Carde and Minks, 1995; Il'ichev et al., 2007; Witzgall et al., 2008), the control of these tortricids largely relies on insecticide sprays (Giner et al., 2012; Knight and Light, 2013). Notwithstanding, the control of CM by means of insecticides is threatened by the widespread development of resistance (Ioriatti et al., 2007; Knight, 2010; Reyes et al., 2007; Rodríguez et al., 2011). Moreover, the current revision of directive on pesticides in the European Community (Directive 128/2009/CE in EU) is strongly reducing the number of active ingredients allowed in many European countries and could enhance the resistance problems.

Spinetoram is a semi-synthetic spynosin insecticide derived from a fermentation product of the actinomycete *Saccharopolyspora spinosa* Mertz et Yao (Mertz and Yao, 1990). The mechanism of

action (IRAC MoA Group 5) involves the disruption of nicotinic acetylcholine receptors (nAChR) and gamma amino butyric acid GABA-gated chloride channels (Dripps et al., 2008; Kirst, 2010). Spinetoram is a broad-spectrum insecticide active against several insect pests in the orders Coleoptera, Diptera, Hemiptera, Lepidoptera, and Thysanoptera (Bacci et al., 2016). Spinetoram is undergoing registration in Europe for its use in fruit and olive orchards. In 2015 the Spanish Ministry of Agriculture granted an exceptional authorisation for using spinetoram to control *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) on cherries and against Psyllidae in pear orchards.

This study aimed to evaluate the efficacy and the residual activity of spinetoram on CM and OFM neonate larvae in semi-field experiments. Laboratory trials were also carried out to assess the dose-mortality relationships and speed of action. These data are necessary to indicate an appropriate range of doses to be used in the field to control the two pests on stone and pome fruits.

2. Materials and methods

2.1. Insects

The populations of *G. molesta* and *C. pomonella* used in the experiments were provided by University of Lleida (Spain) and were reared at the Department of Agricultural Science (University of Bologna, Italy). Up to one hundred generations of both species have been continuously reared on the same artificial diet (Pons et al., 1994) in laboratory conditions (25±1 °C, 70-80% RH and 16/8 L/D). Only active neonate larvae (<24-hour old) were used for all the experiments.

2.2. Insecticides

A commercial formulation of spinetoram (Delegate® 25 WG) was provided by Dow AgroScience Indianapolis, IN. Emamectin benzoate was chosen as a chemical reference because this insecticide is used against Lepidoptera larvae and recommended in IPM of both CM and OFM (Ioriatti et al., 2009). A commercial formulation of emamectin benzoate 0.95% (Affirm® Syngenta Crop

79 Protection Inc.) was purchased. Spinetoram and emamectin benzoate were evaluated both in
80 laboratory and in semi-field trials as commercial wettable granules (WG) formulation (Tab. 1).
81
82 *2.3. Semi-field trials*
83 The insecticides were applied by a backpacked sprayer until run off using a water volume of 12
84 hl/ha. The nectarine orchard was sprayed on July 9th 2013, while the apple orchard was sprayed on
85 July 14th 2014. Spinetoram was tested at three concentrations ranging from 2.5 g a.i./hl to 10 g
86 a.i./hl, emamectin benzoate was applied at the maximum recommended field dose (2.85 g a.i./hl)
87 (Tab 1). The control plots were sprayed with tap water. OFM and CM larval mortality was assessed
88 on nectarines and apples collected from treated orchards. The nectarine orchard was located in the
89 Ravenna district (44° 14' 19''N, 11° 56' 16'' E), its main features were: cv. August Red, 11-yr old,
90 palmette trained, 4.0 x 1.4 m planted, N/S oriented, plants were approximately 4 m high. The apple
91 orchard was located in Bologna district (44° 42' 44'' N, 11° 33' 47'' E) and its main features were:
92 cv. Imperatore, 10-yr old, palmette trained, 3.5 x 1.0 m planted, N/S oriented, plants were
93 approximately 3 m high. Each treatment was applied to three plots randomly assigned within the
94 orchard; the plots consisted of three and five plants for nectarine and apple, respectively. Samples
95 of 10 fruits were collected from plots 3, 7, 14 and 21 days after treatment (DAT). Fruits were
96 carefully inspected for OFM or CM eggs and larvae, before being taken to the laboratory. The main
97 weather conditions during the field trials were: T (mean) = 25.1 °C; RH = 61.4% and 22 mm of rain
98 in 2013 and T = 23.2 °C; RH = 70.2% and 78.80 mm of precipitations in 2014. The highest
99 amounts of rain were recorded at 12 DAT (July 26th, 22.8 mm), at 16 DAT (July 30th, 19.6 mm) and
100 at 20 DAT (August 3rd 14.0 mm).
101 In the laboratory, fruits were separately placed in plastic cups. A neonate larva was then transferred
102 onto each fruit using a fine paintbrush, and cups were closed with lids allowing air circulation. The
103 cups were kept in a climatic chamber at 25 ± 1 °C, 75% RH and 16/8 h L/D photoperiod. The fruits
104 were examined and dissected 10-14 days later, thus allowing the larvae to produce detectable

105 damage. The number of damaged fruits was recorded, and treatments were compared taking into
106 account the percentage of damaged fruits.
107
108 *2.4. Laboratory trials*
109 Laboratory trials were carried out by the surface-treated diet method (Bosch et al., 2007; Reyes and
110 Sauphanor, 2008; Rodríguez et al., 2011). The same meridic diet containing, wheat germ, cereal
111 flours, brewer's yeast, dried apples, agar-agar and preservatives (Pons et al., 1994) was used for
112 both OFM and CM larvae.
113 The insecticide doses used in the lab corresponded to a ten-fold dilution of the doses applied in the
114 field. Spinetoram was tested at concentrations ranging from 0.125 to 1 g a.i./hl to investigate the
115 dose response relationship. Emamectin benzoate was applied at only one rate (0.285 g a.i./hl) as a
116 chemical reference in the trials for the assessment of the speed of action (Tab. 1).
117 The insecticide dilutions were homogeneously distributed on the surface of the diet with a
118 previously humidified paintbrush at a dose of 2 µl/cm^2. After drying the diet was cut in 1-cm^3
119 pieces that were individually placed in plastic jars, then a neonate larva was transferred on the diet
120 surface. Larvae were isolated in a gelatine capsule (Ø 0.5 cm) inserted into the diet, to ensure the
121 contact with the treatment and to avoid escaping. The gelatine capsule was removed 24 h after
122 treatment. Insects were kept at 25 ± 1 °C, 70–80% RH and 16/8 h L/D photoperiod.
123 Mortality was tallied at 24 h and 96 h after treatments and at adult emergence. Given that larvae
124 usually burrow in the diet, it was necessary to dig into the diet pieces to find them for the
125 assessment of mortality. This implies an alteration of the surface test procedure. Therefore, an
126 independent destructive sampling design was used, testing each replicate only once. Four
127 independent replicates with 10 larvae each were carried out for each treatment and for each
128 mortality checking time.
129 Larvae unable to respond to probing with a fine paintbrush were counted as dead.
130

131 *2.5. Statistical analysis*

132 In semi-field trials, linear regression was used to examine the percentage of damaged fruits as a
133 function of DATs for each treatment.

134 The concentration-mortality relationships were analysed by probit model. Only concentrations
135 between the lowest causing >0% mortality and the highest <100% mortality were used. LC_{50} values,
136 95% fiducial limits and slopes of the regression lines were calculated for both species.

137 Speed of action was analysed by factorial ANOVA considering treatments and mortality checking
138 time as a factor. To take into account the natural increase of mortality over time, Abbott corrected
139 *arcsin*-transformed mortalities (Abbott, 1925) were used as the dependent variable. If significant
140 differences were detected by ANOVA, the factors were separated by Ryan-Einot-Gabriel-Welsch-Q
141 (REGW-Q) multiple comparison test. Statistical analyses were carried out using SPSS ver. 13.0
142 (Statistical Package for Social Science, USA) and Statistica Ver. 7.1 (Statsoft Italia).

143

144 **3. Results**

145 *3.1. Semi-field trials*

146 The percentages of nectarines damaged by OFM larvae as a function of DAT showed significant
147 positive linear relationship for all spinetoram doses. The regression analysis of control and
148 emamectin benzoate was not significant, although displaying an overall increase of damage over
149 time. In all the DATs nectarines treated with the highest field dose of spinetoram (10 g a.i./hl) were
150 less injured respect to fruits treated with emamectin benzoate (2.85 g a.i./hl) (Fig. 1; Tab. 2).

151 Regression analysis for CM indicated a significant increase in damage over DAT for all the
152 insecticide treatments, including emamectin benzoate, whose activity declined more sharply respect
153 to spinetoram (Fig. 1; Tab. 2). All the spinetoram doses showed very similar slopes. The
154 interpolation model predicted less than 20% damage for the highest spinetoram dose until 10 DAT
155 approximately.

156 The activity of emamectin benzoate differed between the two species: the percentage of damage
157 caused by OFM was not dependent on DAT, whereas for CM the damage increased sharply as a
158 function of time.
159 Overall many larvae failed to penetrate the untreated fruits (40.0% for OFM and 29.2% for CM).
160 The inability of neonate larvae to enter unripen fruits was particularly marked for *G. molesta*, and it
161 is well known in field condition (Blomefield and Giliomee, 2012; Westigard et al., 1976).
162
163 *3.2. Laboratory trials*
164 The probit model significantly fitted the dose-response curves for both species (Tab. 3). Although
165 the LC_{50} value of spinetoram was approximately 28% higher for *C. pomonella* (8.44 ng a.i./cm^2)
166 compared to *G. molesta* (6.59 ng a.i./cm^2), the slope was steeper for CM.
167 The Abbott-corrected mortality of *G. molesta* did not increase over exposure time (Fig. 2A) and it
168 can be assumed that the lethal effect occurred during the first 24 h. On the other hand, *C. pomonella*
169 corrected mortality increased as a function of exposure time ($p = 0.018$) (Fig. 2B). The multiple
170 comparison REGW-Q test identified a significant difference only between 24 h and adult
171 emergence. The increase of mortality for the lower spinetoram doses (0.125 and 0.25 g a.i./hl) was
172 more marked from 96 h to adult emergence. Conversely, for spinetoram at 0.5 g a.i./hl and for
173 emamectin benzoate the main increase was between 24 and 96 h. Spinetoram at 1 g a.i./hl caused
174 97.5% corrected mortality within 24 h and reached to 100% both at 96 h and at adult emergence.
175
176 **4. Discussion**
177 The laboratory trials revealed similar activity of spinetoram on both OFM and CM larvae, even if
178 OFM was slightly more susceptible to lower doses. The difference in behaviour and size of neonate
179 larvae of the two species could explain this finding. OFM larvae are less strictly internal feeders and
180 are known to forage on fruits or tree shoots for a longer period than CM larvae, which in contrast

usually enter the fruits within a few hours of egg hatching (Van Emden, 2013). As a result, the OFM larvae may have longer contact period with the treated surfaces, thus uptaking higher amounts of insecticides. Also the size of the neonate larvae may have a certain relevance: indeed OFM 1st instar larvae are smaller (1.4 mm) than CM larvae (2.3 mm) (Balachowsky, 1972). Therefore, it is likely that a lower dose is needed to kill the former than the latter.

The results of the semi-field trials showing longer activity on OFM than on CM are in agreement with lab probit regressions, as low residual amounts of a.i. probably remain on field-aged fruits. It is to be pointed out that in 2014 three rain events were recorded in the second half of the field trials in the apple orchard. Wash-off may have contributed to the loss of efficacy of spinetoram on CM.

Overall our data indicate that the recommended field dose for spinetoram should be between 5 and 10 g a.i./hl depending on the infestation rate, pest generation and orchard type. In comparison with the chemical reference (emamectin benzoate), spinetoram at the highest field dose (10 g a.i./hl) displayed longer activity, achieving higher control at all DAT on both species. At 5 g a.i./hl spinetoram showed a performance similar to emamectin benzoate.

At 24 h spinetoram showed remarkable speed of action on both species. This was not surprising because, like other spinosyn insecticides, spinetoram is a nerve poison that operates quickly through disruption GABA-gated chloride channels (Orr et al., 2009). Given that both species do not ingest fruit skin while tunnelling (Balachowsky, 1972; Ioriatti et al., 2009), the major mode of insecticide uptake seems by contact.

The semi-artificial diet containing dried apple (Pons et al., 1994) was suitable for rearing both species and the methodology used for testing neonate larvae was also appropriate as no differences were detected between mortalities on untreated diet at any mortality checking interval.

The LC_{50} values were lower for OFM than for CM, and this is in line with the results of other studies reporting a higher toxicity of spinetoram on *G. molesta* (Jones et al., 2010; Magalhaes and Walgenbach, 2011). In particular, Magalhaes and Walgenbach (2011) reported LC_{50} values for surface-treated assays of 0.06 μg a.i./ml and 0.05 μg a.i./ml for CM and OFM, respectively.

Considering the differences in insect strains, in the kind of diet used, in the amount of the insecticide solutions applied to the diet surface and the conversion from μg a.i./ml to ng of a.i./cm^2, our data are in the same order of magnitude.

In conclusion, our results indicated spinetoram as a valuable candidate to be used in multi-strategy IPM control programs in pome and stone fruit orchards. In particular, spinetoram could be scheduled after an ovicide spray against CM 1st larval generation because its residual activity provides a satisfactory level of control for approximately 10 days after treatment. The speed of action and the high activity at 10 g a.i./hl might be key requirements to control the summer CM generations. Similar considerations can be drawn for the management of OFM on nectarines. Depending on the weather and the agronomic conditions, OFM can cause occasional damage also to pome fruits. In these cases spinetoram sprays, being active on both OFM and CM, could be useful to control both species with a single insecticide application.

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Tables

Table 1. Insecticides and concentrations used in semi-field and laboratory experiments on neonate larvae of *Grapholita molesta* and *Cydia pomonella*. WG = Wettable Granules

Commercial name	Active ingredient	Semi-field trial doses (g a.i./hl)	Laboratory trial doses (g a.i./hl)
Delegate 25 WG	spinetoram		0.125
		2.5	0.25
		5	0.5
		10	1
Affirm 95 WG	emamectin benzoate	2.85*	0.285
untreated control	-	tap water	distilled water

* Maximum recommended field dose

Table 2. Parameters of the linear regressions of the percentage of damaged fruits as a function of the days after treatment in the semi-field assay.

	R^2	Adjusted R^2	F (1, 10)	p	Regression line equation
Grapholita molesta					
Control	0.159	0.075	1.90	0.1985	
Emamectin benzoate 2.85 g a.i./hl	0.226	0.148	2.91	0.1186	
Spinetoram 2.5 g a.i./hl	0.570	0.527	13.25	0.0045	y = 13.7748+2.3311x
Spinetoram 5 g a.i./hl $^{-1}$	0.622	0.584	16.46	0.0023	y = 4.3488+2.4283x
Spinetoram 10 g a.i./hl	0.531	0.484	11..31	0.0072	y = -2.8477+2.0309x
Cydia pomonella					
Control	0.282	0.210	3.93	0.0756	
Emamectin benzoate 2.85 g a.i./hl	0.925	0.918	123.68	< 0.0001	y = -5.4084+4.777x
Spinetoram 2.5 g a.i./hl	0.790	0.769	37.69	0.0001	y = 8.9183+3.3554x
Spinetoram 5 g a.i./hl $^{-1}$	0.796	0.776	39.14	0.0001	y = -5.298+3.3598x
Spinetoram 10 g a.i./hl	0.711	0.682	24.56	0.0006	y = -10.6402+3.0199x

Table 3. Dose-response statistics for the surface-treated diet bioassay with spinetoram on neonate larvae of *Grapholita molesta* and *Cydia pomonella*. Mortality was checked at 24 h, insecticide amount was expressed as ng of active ingredient per cm^2.

Species	N	Slope (SE)	LC_{50} (ng a.i./cm^2)	95% fiducial limit (ng a.i./cm^2)	χ^2	p
G. molesta	200	2.53 (0.93)	6.59	0.47 - 10.95	1.03	0.309
C. pomonella	200	4.42 (1.14)	8.44	5.20 - 10.61	0.97	0.325

Figure(s)

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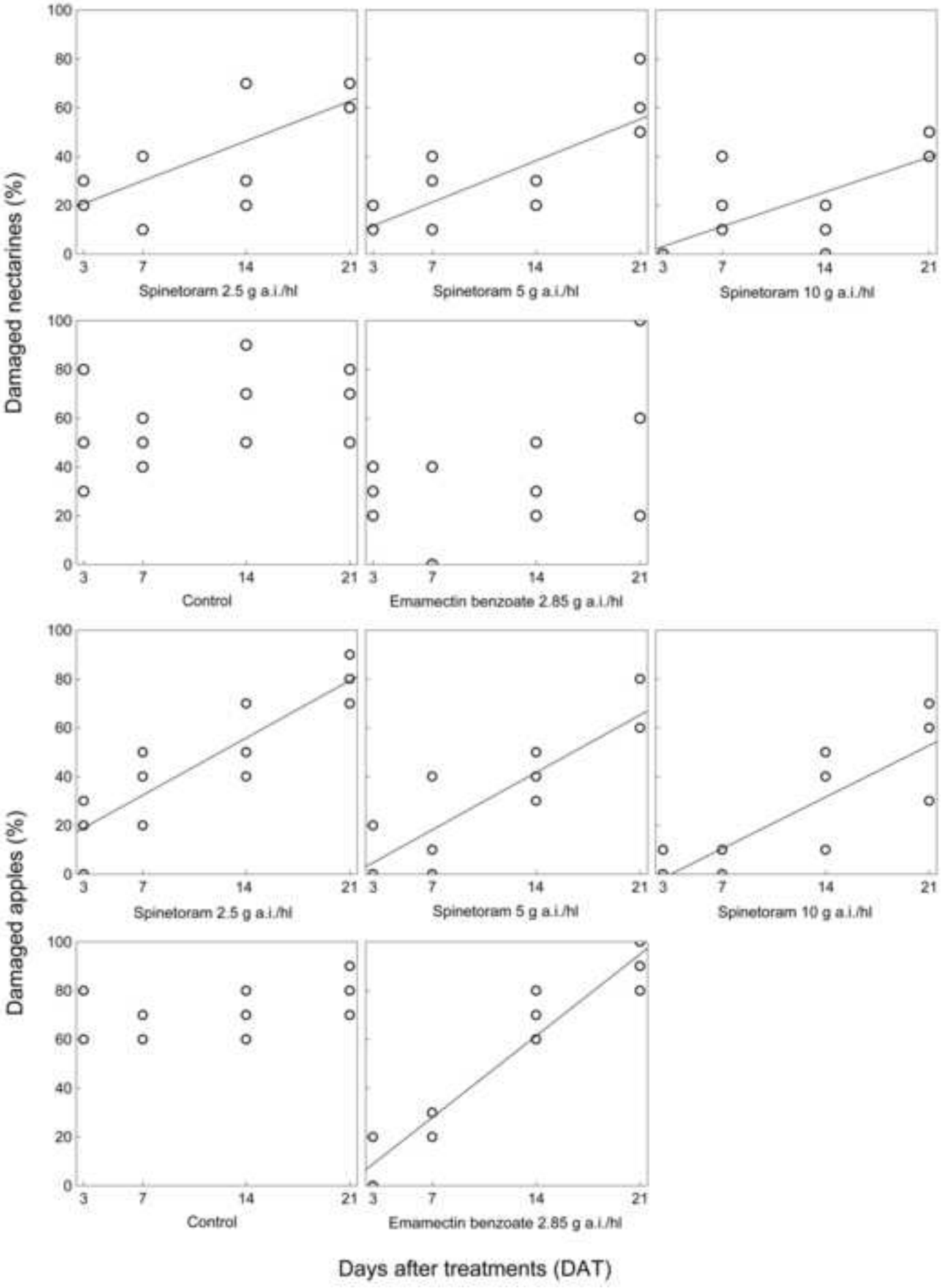

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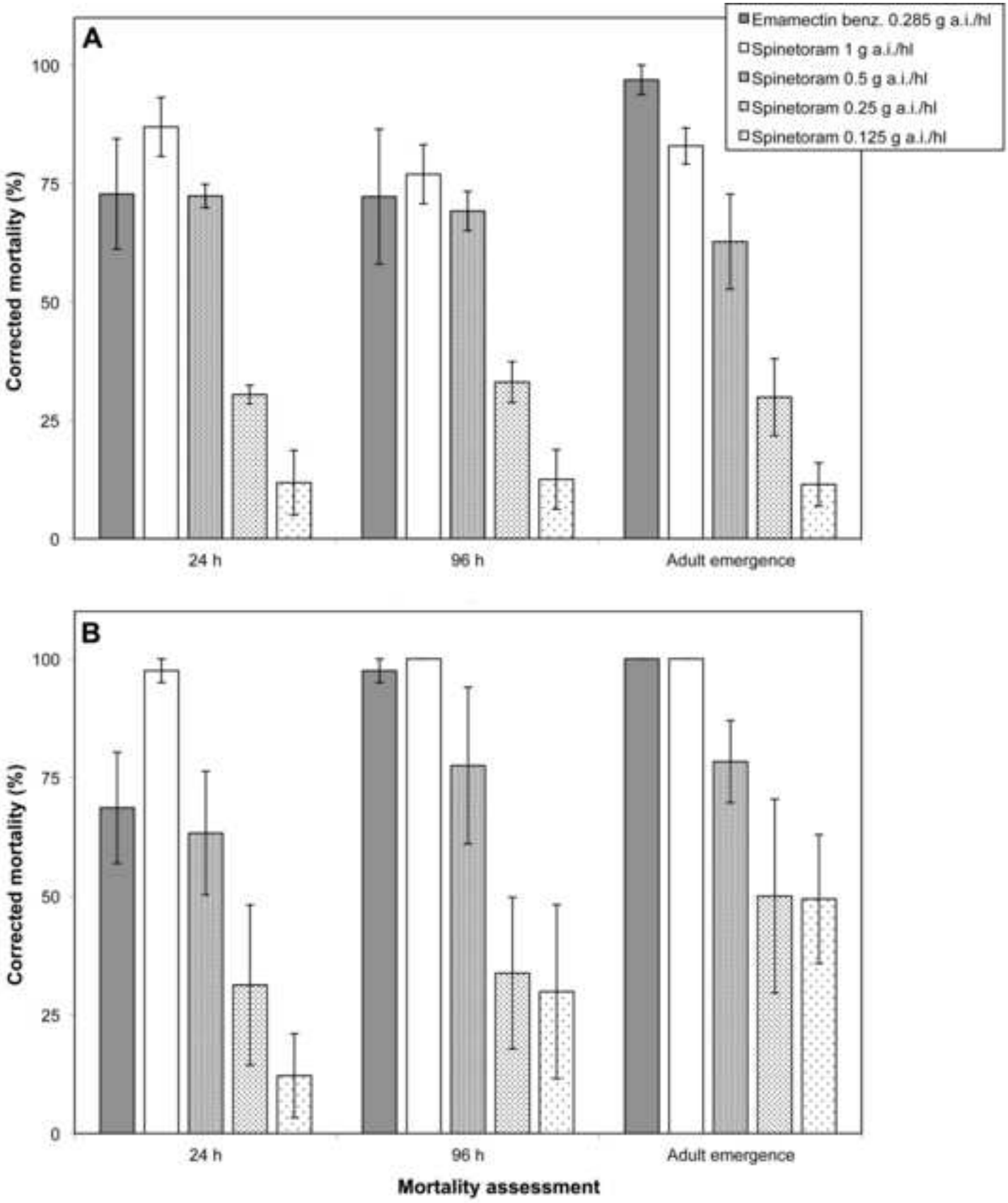

Figure captions

Figure 1. Linear regression of the percentages of damaged fruits as a function of days after treatment. A) *Grapholita molesta* neonate larvae tested on treated nectarines. B) *Cydia pomonella* neonate larvae tested on treated apples.

Figure 2. Abbott corrected mortality of *Grapholita molesta* [ANOVA F (2, 45) = 0.739, p = 0.483; A] and *Cydia pomonella* [ANOVA F (2, 45) = 4.407, p = 0.018; B] evaluated at three different times. Bars represent the standard errors of means.

Highlights

The susceptibility of *Grapholita molesta* and *Cydia pomonella* to spinetoram was tested on neonate larvae.
Semi-field and laboratory trials showed efficacy on both species.
Spinetoram might be considered a valuable candidate in multi-strategy IPM control programs in pome and stone fruit orchards.